THE MECHANISM OF AGGLUTINATION.

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Pfeiffer and his pupils about 1894 discovered that when an animal is repeatedly inoculated with certain organisms, its serum has the power of causing the organisms in a bouillon culture to become altered and to cohere or agglutinate into microscopical masses or clumps. The serum only reacts in this manner or is active with the bacteria with which the animal has been inoculated, and this fact caused the reaction to be used as a diagnostic for that particular organism. Widal inverted the reaction and used a culture of typhoid bacteria to discover whether or not a serum was active, and especially in human practice to determine if a patient had typhoid fever. To apply the test a drop or loop of the blood serum which has separated from the clot is added to about 30 drops or loops of bouillon containing typhoid bacteria. Should the bacteria collect into clumps in half-an-hour the reaction is positive, and is by some considered as a proof of typhoid fever, by others (1) as a symptom of that disease. The test has been extended to other diseases.

The phenomenon of agglutination forcibly recalls that of flocculation or coagulation of inorganic particles, where instead of adding an indefinite active serum there is added a definite chemical substance, and a natural assumption would be that they are brought about by the same causes. One difference, however, between the two phenomena is that the bacteria are living and sensitive, while inorganic particles are insensitive. Before the act of agglutination they are actively motile; the agglutinine in the serum causes them to lose their motility in great part or entirely; they become immobilised. After a variable time, it may be hours or days, the bacteria regain their motility and the clumps break

up. It is clear that the loss of motility or suspension of vitality is a necessary factor in the phenomenon.

The action of the serum upon the bacteria has been variously explained. Pfeiffer saw the bacteria swell just as they did in the peritoneal fluids of immune animals, and Gruber (2) considered this to be the cause of agglutination, the outer membranes of the bacteria becoming gelatinous and sticking to one another.

Kraus (3) obtained a precipitate on adding anti-cholera serum to a filtered culture of cholera vibrions. This is an agglutination of the soluble products of the metabolism or of the disintegration of the bacteria. Nicolle (4) showed that these agglutinable substances were excreted by the bacteria during life as well as being contained in the products of their disintegration. These bodies are not affected by a temperature of 150° C. When inert chemical substances such as talc, or as Nicolle showed, foreign bacteria are introduced into the filtured culture they are entrapped in the precipitate produced by the active serum and appear to agglutinate. Nicolle and also Paltauf considered that this gelatinous precipitate surrounded the bacteria and caused them to adhere together. Dineur considered that the precipitation took place on the flagella, which becoming adhesive caused an entanglement of the bacteria.

In reviewing these hypotheses Bordet (5) considered that even if the membranes were altered there was no reason adduced for the bacteria becoming attracted to one another. He considered that Kraus' precipitate was too slowly formed to account for the rapid agglutination of the bacteria, and furthermore that the coagulum upon the surface of the bacteria was only an idea and had not been demonstrated. Bordet's objections seem to be rather weak, for it is a well known fact that substances about to separate out from a liquid do so rapidly when small particles are present in the liquid, and there is every probability that there will be a film of the precipitated substance upon the flagella and upon the surfaces of the bacteria long before a precipitate becomes visible in the fluid.

Bordet considers that the cause of agglutination is also the cause of the coagulation of casein, of the precipitation of chemical substances and of the agglutination of blood corpuscles. To disprove Gruber's hypothesis that the swelling of the bacterial capsule causes agglutination, he added a small quantity of an active serum to a suspension of cholera vibrions in normal saline. The clumps which formed were separated from the normal saline by centrifuging and subsequent treatment with water. The bacteria were shaken with the water until a homogeneous suspension was obtained. This was divided into two portions, to one of which common salt solution was added and to the other distilled water. Clumping occurred in the former case, but not in the latter. That common salt should cause the agglutination of the immobilised bacteria shows that a swelling of the membranes, if such occur, is not a necessary factor in the phenomenon. It is contended by Bordet that the agglutination of bacteria by active sera is identical in principle with the coagulation of casein by rennet. In the furtherance of this idea he found that the serum of animals inoculated with milk contained an enzyme that coagulated milk after the manner of rennet. Since both phenomena appear to be similar, he considered that the name agglutination should be changed to coagulation, and the agglutinines, of which there are many varieties—each capable of clumping its particular organisms—should be called coagulines. The agglutinines he believes to be enzymes, an opinion which is shared by Emmerich and Löw (6). The mechanism of the process, as explained by Bordet, consists primarily in the enzyme altering the relations between the bacteria and the solution, and secondly, as a result of the alteration the bacteria gather themselves into clumps.

It is claimed by those who have experimented with the mechanism of agglutination that clumping is caused by a precipitate forming on the organisms (Nicolle, Paltauf) and making them adhesive, by the organisms swelling (Gruber), or by the agglutinating enzyme causing them to flocculate (Bordet). Neither the formation of a precipitate on the bacteria, as Bordet has

pointed out, nor the swelling of the organism explains the reason of their gathering together. With regard to the action of the enzyme in causing them to run together, Bordet has shown that once the bacteria have been acted upon by the active serum they are flocculated by common salt. It has been shown in my former paper that bacteria are not flocculated by common salt, from which it is to be concluded that the organisms have through the action of the active serum become altered into or have been endowed with some substance that is capable of being coagulated or flocculated. That it is not the action of the enzyme purely, is shown by Bordet's experiment, but as I shall show he has wrongly interpreted the phenomenon. He undoubtedly considers agglutination to be the work of the enzyme alone and confirms it by the action of rennet on milk, apparently forgetting that rennet does not coagulate casein in the absence of salts of lime. The casein is altered by the rennin into paracasein and an albumose, but the paracasein is only coagulated in the presence of lime.

The question then arises, what is the action of the active serum? It is apparently not a coagulation of the protoplasmic albuminoids, since bacteria, the albumen of which has been coagulated by heat, are, as I have found, not flocculated by salts. The immobilisation would seem to indicate an alteration of the protoplasm. But since bacteria killed by heat are not flocculated, it does not seem probable that any alteration which the protoplasm might undergo would induce agglutination. We must therefore look to an agglutinable substance being formed on the bacteria. Since Kraus' precipitate is formed by the active serum sooner or later in the fluid in which the bacteria have been grown, there can be no doubt that the bacteria are saturated with the precipitable substance before it diffuses into the fluid. The precipitate would naturally form upon or in the bacteria very much sooner than in the medium. It would appear first upon the delicate flagella, which in their motion would strike one another and on doing so would adhere. The motility would accordingly cease, and the precipitate, having by this time formed on the body of the organism, would be flocculated by the salts of the

bouillon or serum. *It is the precipitate that is clumped; the bacteria are carried with it mechanically.* By adopting this view we have an agreement between the numerous observers. It is also easily understood why dead typhoid bacteria agglutinate like living ones. The products of metabolism being in contact with the dead cells are precipitated by the active serum upon their surfaces and in the medium. The precipitate is flocculated by the saline constituents of the solution, and both dead cells and precipitate gather together into floccules. The fact that dead typhoid bacteria may be employed in Widal's test bears out the theory of a coagulable surface precipitate, and agrees with Nicolle's experiments, which showed that foreign bacteria in a filtered culture of *Bact. typhi* were clumped by active typhoid sera.

Gruber (7), writing recently, considers that Kraus' precipitate is quantitatively too small to explain agglutination, and thinks it probable that in the act of agglutination certain substances in the bacterial membranes are made more insoluble. A shrinkage and separation follow whereby glutinous masses are formed on the bacterial surfaces. This appears to be very similar to his old hypothesis of the swelling of the membranes, to which Bordet pointed out that there was no reason given for the approach of the bacteria. Again, the shrinkage and formation of sticky masses on the bacteria is an idea, while Kraus' precipitate is a fact.

Radzievsky (8), in a preliminary paper, objects to the precipitate idea apparently because he obtained no precipitate in young cultures in which the bacteria clumped normally. Both writers apparently forget that the bacteria must be saturated with the precipitable substance before it is given off into the culture medium, and in a young culture while the organisms are saturated there may be but an infinitely small amount in the culture fluid.

I have tested the validity of Gruber's and of Radzievsky's objections and cannot agree with them. The precipitate may be and undoubtedly is very small in amount, but it is still appreciable. A twenty-four hours' bouillon culture of *Bact. typhi* was filtered through a Kitasato filter, and an agar culture of *Bact.*

coli commune was distributed in a small portion of the filtrate. This suspension was treated with active typhoid serum in the proportion of 20 parts of suspension to 1 of the serum. The bacteria, which before the addition of the active serum had been uniformly distributed in the *Bact. typhi* filtrate, had after an hour become collected into clumps. As it seemed possible that some objection might be made to the use of *Bact. coli commune* on the ground that the serum might have been obtained from a case of mixed *Bact. typhi* and *Bact. coli commune* infection, a second experiment was made with *Bact. Hartlebii*. Agglutination occurred precisely as when *Bact. coli commune* had been employed. These experiments show that Gruber's and Radzievsky's objections are groundless, and they are in agreement with Nicolle's, who showed that foreign bacteria suspended in the filtrate of a *Bact. typhi* culture were agglutinated by active sera.

Since agglutination is essentially the coagulation of a precipitate, it will be prevented by the presence of anti-coagulating agents such as the alkaline citrates and acetates. Winterberg (9) in a recent paper showed that the so-called agglutinines were destroyed by acetates, as evidenced by the absence of clumping. It is clear that the non-clumping was due to the acetates preventing the flocculating action of the serum and bouillon salts and not to the destruction of the enzyme.

Although bacteria are not flocculated by salts like inorganic particles, it seemed possible that they might be induced to simulate flocculation by causing a silver compound to be formed upon the outer surface of the cells. With this object in view, bouillon cultures of *Bact. typhi*, *Bact. coli commune*, and *Bact. prodigiosum* were filtered through Kitasato filters, washed with sterile distilled water and finally suspended in distilled water. Again, cultures of these three organisms were scraped from an agar surface and suspended in distilled water. All the suspensions were gently centrifuged to eliminate clumps and to obtain a uniform suspension of the bacteria. The suspension was then treated with a few drops of 0·5 % silver nitrate (a quantity which was in excess as far as chlorides were concerned) and centrifuged

to eliminate from the fluid traces of precipitable salts. Generally a slight precipitate was obtained, and above this a uniform suspension of the bacteria. The nitrates of potash, soda and ammonia failed to produce a flocculation when added to the suspension, even when the emulsions were centrifuged (2,500 revolutions per minute). The continued addition of dilute silver nitrate in place of the alkali nitrates produced very slight precipitates. Strong silver nitrate, however, produced complete precipitation.

The failure of the dilute silver nitrate to effect complete flocculation shows that either no silver salt had formed on the surfaces of the bacteria, or, if one had formed, the silver nitrate or the alkali nitrates were too weak to induce flocculation. It is probable that no surface film had been formed. The absence of flocculation by so strong a flocculating agent as dilute silver nitrate emphasises the fact that bacteria when freed from bouillon salts and their by-products, are not coagulated like inorganic particles.

The bacteria after being flocculated by the strong silver nitrate were seen to be in clumps. Careful examination of these clumps, and especially after they had been exposed to the light, showed that the bacteria were enclosed in a matrix which undoubtedly consisted of a silver compound of the intracellular salts which had diffused out from the cells under the influence of the strong silver nitrate. It is evidently impossible to obtain a pure flocculation or agglutination of bacteria, and when such an appearance is presented the failure to reveal the presence of a flocculated matrix is due entirely to our instruments or methods of demonstration.

Some experiments of Malvoz (10) are frequently quoted to show that agglutination of typhoid bacteria may be obtained by the addition of certain chemical reagents and stains. Since I have shown that true chemical agglutination does not occur, it seemed advisable to repeat his experiments. In one of these experiments clumping occurs when strong alcohol or strong formalin is added

to an equal volume of a suspension of bacteria in water. This appears to be due to a dehydration rather than to a flocculation in which the loose water molecules are withdrawn. But such as it is, these strong reagents produce the nearest approach to true flocculation that can be obtained with bacteria. Another of his agglutinating agents is dilute mercuric chloride. This salt undoubtedly acts like calcium chloride in producing a precipitate of the culture salts that is flocculated together with the bacteria. The case of a dilute solution of saffranin promised to be different. This stain when in dilute solution (1-1000) and added to an equal volume of bacterial suspension produced an apparent agglutination of the bacteria. A test of the stain, however, with sterile bouillon showed the formation of an immediate precipitate which was found microscopically to resemble clumps of bacteria and cocci. This shows that the case of saffranin is no exception to the rule that in agglutination a precipitate is first formed in the fluid. On investigating the constituent of the bouillon that is precipitated by saffranin, it was found to be among those that are precipitated by lime, since no agglutination was obtained with bacteria that had been grown upon or in media that had been treated with lime to remove phosphoric acid. It does not appear to be a phosphate, because neither ammonium nor potassium phosphate forms a precipitate with the dilute stain. Malvoz ascribes the coagulating effect of dilute alkalies to the formation of calcium carbonate. I have already shown that is due to the formation of calcium phosphate.

In conclusion, it appears that agglutination is caused by the formation of a delicate precipitate on the outer surfaces of the bacteria and in the fluid in which the bacteria are suspended. This precipitate is flocculated or coagulated by the saline constituents of the medium and of the serum. Since the precipitate is invisible to ordinary microscopical observation and the bacteria are visible, an apparent agglutination of the latter only is seen to take place by the action of active sera.

LITERATURE.

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4. —Nicolle, Ann. Institut Pasteur, xii. 161.
5.—Bordet, Ann. Institut Pasteur, xiii. 225.
6.—Emmerich and Löw, Zeitschr. für Hygiene, xxxii. 1.
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9.—Winterberg, Zeitschr. für Hygiene, xxxii. 375.
10.—Malvoz, Ann. Institut Pasteur, xi. 582.